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Conservation of functional features of U6atac and U12 snRNAs between vertebrates and higher plants

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ABSTRACT

Splicing of U12-dependent introns requires the function of U11, U12, U6atac, U4atac, and U5 snRNAs. Recent studies have suggested that U6atac and U12 snRNAs interact extensively with each other, as well as with the pre-mRNA by Watson–Crick base pairing. The overall structure and many of the sequences are very similar to the highly conserved analogous regions of U6 and U2 snRNAs. We have identified the homologs of U6atac and U12 snRNAs in the plant *Arabidopsis thaliana*. These snRNAs are significantly diverged from human, showing overall identities of 65% for U6atac and 55% for U12 snRNA. However, there is almost complete conservation of the sequences and structures that are implicated in splicing. The sequence of plant U6atac snRNA shows complete conservation of the nucleotides that base pair to the 5′ splice site sequences of U12-dependent introns in human. The immediately adjacent AGAGA sequence, which is found in human U6atac and all U6 snRNAs, is also conserved. High conservation is also observed in the sequences of U6atac and U12 that are believed to base pair with each other. The intramolecular U6atac stem-loop structure immediately adjacent to the U12 interaction region differs from the human sequence in 9 out of 21 positions. Most of these differences are in base pairing regions with compensatory changes occurring across th stem. To show that this stem-loop was functional, it was transplanted into a human suppressor U6atac snRNA expression construct. This chimeric snRNA was inactive in vivo but could be rescued by coexpression of a U4atac snRNA expression construct containing compensatory mutations that restored base pairing to the chimeric U6atac snRNA. These data show that base pairing of U4atac snRNA to U6atac snRNA has a required role in vivo and that the plant U6atac intramolecular stem-loop is the functional analog of the human sequence.

Keywords: evolution; pre-mRNA splicing; spliceosomes; U12-dependent introns

INTRODUCTION

The presence of multiple introns in most nuclear mRNA coding genes is a distinctive feature of the genomes of animals and higher plants. The consensus features of splice sites in these two groups of organisms are very similar, although there may be some differences in the mechanism of splice site definition (Wiebauer et al., 1988; Simpson & Filipowicz, 1996; Brown & Simpson, 1998). Even more striking is the conservation of the sequences and structures of the small nuclear RNAs that are involved in spliceosome structure and function (Brow & Guthrie, 1988; Guthrie & Patterson, 1988; Reddy & Busch, 1988). The most conserved regions of these snRNAs are the portions of U6 and U2 that are believed to comprise the central set of RNA–RNA interactions in the spliceosome (Nilsen, 1998). In these regions, the sequences of human and plant snRNAs are almost identical. Although this conservation of sequences over the billion years of evolution that separate these taxonomic groups testifies to their important function in splicing, the lack of variation makes it difficult to use phylogenetic covariation to substantiate potential RNA–RNA interactions.

The recent identification of a minor class of nuclear pre-mRNA introns that are spliced by a distinct alternative spliceosome has provided an unexpected example in which to evaluate the present models of RNA interactions in the spliceosome (reviewed in Tarn &

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Steitz, 1997; Burge et al., 1998b; Nilsen, 1998). These introns were first identified exclusively in animals (Jackson, 1991; Hall & Padgett, 1994) and the splicing pathway and snRNA components were initially identified in extracts of human cells (Tarn & Steitz, 1996a, 1996b). Subsequently, it was discovered that introns with identical consensus splice site features are present in plants (Wu et al., 1996). This suggested that the origins of this class predated the divergence of the plant and animal kingdoms.

The snRNAs that are involved in splicing this minor (U12-dependent) class of introns in human cells have been shown to be functional analogs of the major (U2-dependent) class spliceosomal snRNAs. U11 snRNA appears to be the functional analog of U1 snRNA, U12 snRNA is the analog of U2 snRNA, U4atac snRNA is the analog of U4 snRNA and U6atac snRNA is the analog of U6 snRNA. U5 snRNA appears to function in both spliceosomes (reviewed in Tarn & Steitz, 1997). The functional similarities of the two sets of snRNAs are given added support by the apparent conservation of RNA–RNA interactions between the pre-mRNA splice sites, U12 snRNA, U6atac snRNA, and U11 snRNA (see Tarn & Steitz, 1997; Burge et al., 1998b; Nilsen, 1998).

As part of an effort to understand the structure/function relationships in these newly identified snRNAs, we wanted to carry out a phylogenetic comparison particularly of U12 and U6atac snRNAs because they are thought to be most central to the splicing reactions. U12-dependent introns have been found in fish, amphibians, birds, mammals, insects, jellyfish, and higher plants (Burge et al., 1998a). However, U12-dependent introns do not appear to exist in the yeast *Saccharomyces cerevisiae* or the nematode *Caenorhabditis elegans* based on analysis of the complete or nearly complete genome sequences, respectively (Burge et al., 1998a). Thus, the animal–plant divergence represents the deepest branching known of the U12-dependent introns.

A recent genomic sequence database search identified 11 probable U12-dependent introns out of 19,553 introns in the plant *Arabidopsis thaliana* (Burge et al., 1998a). Remarkably, in almost every instance, the splice site sequences of the plant U12-dependent introns are identical to the animal sequences in spite of the evolutionary distance. The existence of these introns in plants implies the parallel existence of a set of snRNAs to splice them. Here we report on the identification of snRNAs from *Arabidopsis* that appear to be the plant homologs of U6atac and U12 snRNAs. Surprisingly, the sequence conservation of these snRNAs between humans and plants is much less than that seen in the major class snRNAs. This allows us to use phylogenetic covariation to investigate the RNA–RNA interactions that have been proposed to occur in the U12-dependent spliceosome.

RESULTS

Identification of a putative plant U6atac snRNA homolog

A search of plant genomic sequences for similarities to the snRNAs of the human U12-dependent spliceosome revealed a provocative match to the sequence of human U6atac snRNA. This sequence, shown in Figure 1, appears to encode an snRNA of similar length to human U6atac with about 65% sequence identity. The sequence similarity is highest in the 5′ portion of the putative snRNA where the regions of interaction with U12 snRNA and the pre-mRNA 5′ splice site have been localized.

The sequence of the flanking genomic region of this sequence contains elements that would appear to support transcription of this snRNA in plant cells based on their similarity to elements flanking active U6 snRNA genes (Waibel & Filipowicz, 1990). An upstream sequence element (USE) with the sequence GTCCCA CATCG occurs at position −67 to −57 upstream of the putative transcription initiation site in *Arabidopsis* U6atac snRNA. This sequence is identical to USE sequences found in the U6-1 and U6-26 snRNA genes of *Arabidopsis* at position −66 to −56 from the transcription initiation sites (Waibel & Filipowicz, 1990). A second conserved USE is a TATA-like box with the sequence TATATATA at position −32 to −25 in the *Arabidopsis* U6atac snRNA gene. A similar element with the sequence TTTATATA at position −31 to −24 is found in the *Arabidopsis* U6-1, U6-26, and U6-29 snRNA genes. The final USE is a cap-adjacent sequence, GATT, located between −4 and −1, which is conserved in *Arabidopsis* U6atac snRNA and all three U6 snRNA genes of *Arabidopsis*. All three *Arabidopsis* U6 snRNA genes have been shown to be transcriptionally active and resistant to α-amanitin (Waibel & Filipowicz, 1990). These similarities suggest that this *Arabidopsis* U6atac gene is transcriptionally active and is probably transcribed by RNA polymerase III similar to the U6 snRNA genes.

To verify that this RNA was expressed in cells, a sample of total RNA from *Arabidopsis thaliana* was reverse transcribed using a primer derived from the genomic sequence described above. The resulting cDNA was amplified by PCR using the same primer and a primer from the predicted 5′ end of the snRNA. A PCR fragment of the expected size was amplified and cloned that matched the genomic sequence. Amplification reactions carried out without reverse transcription failed to yield a DNA product, confirming that the RT-PCR product was not derived from contaminating DNA (data not shown). As a further test of the expression of this snRNA, a sample of total RNA was separated on a denaturing polyacrylamide gel, blotted to nylon membrane, and probed with the labeled PCR fragment. The probe hybridized to a single RNA of about 125 nt

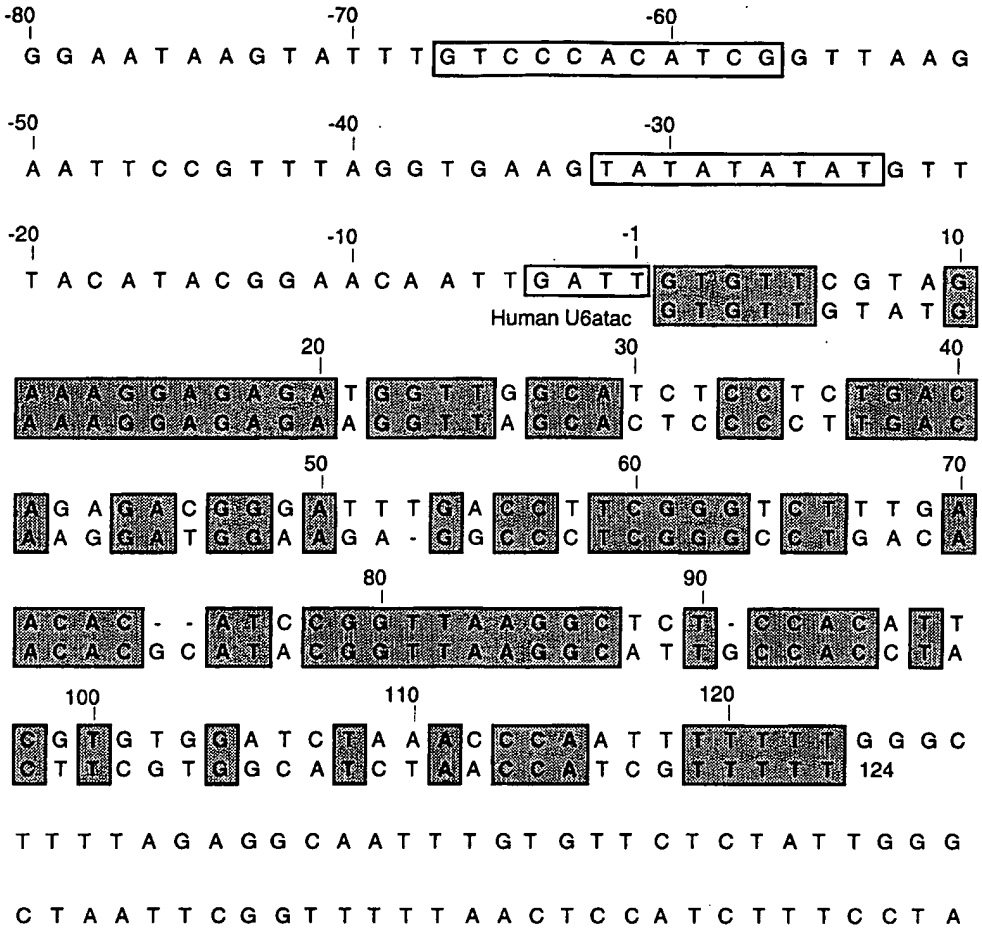

FIGURE 1. Sequence of the *Arabidopsis* U6atac snRNA gene. The conserved promoter elements in the −80 to −1 region discussed in the text are enclosed in open boxes. The human U6atac snRNA sequence is shown beneath the *Arabidopsis* sequence and conserved nucleotide positions are enclosed in shaded boxes. The numbering for human U6atac is used for the snRNA sequences. Gaps introduced into the alignment are shown as hyphens (-). The sequence is from GenBank Accession #AB006702.

(Fig. 2). The sequence of the 5′ and 3′ ends of the U6atac snRNA were subsequently determined by sequencing of cloned 5′ and 3′ RACE products prepared as described in Materials and Methods. The sequences of these clones matched the genomic sequence at the 5′ and 3′ ends. The length of the RNA determined by the RACE procedure is 123 nt. Although this is longer than the U6 snRNAs of human (107 nt) or *Arabidopsis* (102 nt), it is similar to the length of human U6atac snRNA (125 nt). These results establish that the genomic sequence is expressed as a small RNA in *Arabidopsis*.

The putative plant U6atac snRNA homolog can be folded into a hypothetical secondary structure similar to that proposed for human U6atac snRNA (Fig. 3). The 5′ stem-loop structure of the plant RNA is similar in size and position, but appears to be somewhat less stable than that of the human structure. In both RNAs, the critical nucleotides that base pair with the U12-dependent intron 5′ splice site are located in the 5′ loop. The middle stem-loop of the plant sequence appears to be stronger than in human U6atac. These nucleotides, however, are probably base paired with U4atac snRNA in the di-snRNP particle rather than in the structure shown here for the isolated U6atac snRNA (Tarn & Steitz, 1996b). Other foldings can be generated for both the plant and human sequences. In the absence of physical information, the structures shown here are provisional at best.

Identification of a putative plant U12 snRNA homolog

The initial database searches of available plant genomic sequences did not reveal a candidate with a significant match to human U12 snRNA. Based on both the conservation of the branch site sequence in plant U12-dependent introns and the conservation in the plant U6atac snRNA identified above of the putative region of interaction with U12 snRNA, we probed a blot of

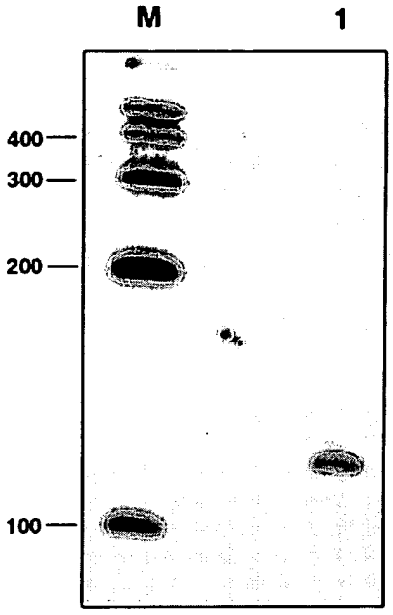

FIGURE 2. Expression of the *Arabidopsis* U6atac snRNA shown by Northern blot analysis of total RNA. Ten micrograms of total RNA from *Arabidopsis* was fractionated on a 8% denaturing polyacrylamide gel and transferred to a nylon membrane. The blot was hybridized with ^{32}P-labeled U6atac snRNA cDNA. The sizes of the ^{32}P-labeled in vitro transcribed RNA markers (Ambion) in nucleotides are given at the left.

Arabidopsis RNA with an oligonucleotide complementary to the branch site and U6atac interaction regions of human U12 snRNA. This probe hybridized to an RNA of approximately 175 nt (data not shown) suggesting the existence of a potential U12 snRNA homolog in *Arabidopsis*.

To determine the sequence of this putative plant U12 snRNA homolog, we employed 3′ RACE on *Arabidopsis* size-fractionated total cell RNA in the 100–200 nt range. This RNA was 3′ polyadenylated using yeast poly (A) polymerase and then reverse transcribed using an oligo d(T) primer. The cDNA was amplified using an oligonucleotide containing nt 1–24 of human U12 snRNA and the oligo d(T) primer. This yielded a DNA fragment of approximately 175 bp, which was cloned and sequenced. To obtain the sequence of the 5′ end of the RNA, a 5′ RACE procedure was employed using an internal primer (see Materials and Methods). The complete sequence of the RNA is shown in Figure 4, aligned with known U12 snRNA homologs (Tarn et al., 1995; Yu et al., 1996). Hybridization to a blot of total *Arabidopsis* RNA using a probe from the complete snRNA identified a single RNA of about 175 nt in agreement with the size of the predicted RNA from the RACE experiments (Fig. 5).

The alignment in Figure 4 shows that this RNA is highly similar to other U12 snRNAs and thus supports the conclusion that this is the plant U12 snRNA. The plant RNA shows 52–59% sequence identity to the other U12 snRNAs. The similarity is strongest in the 5′ end where the regions that interact with the intron branch site and with U6atac snRNA are located. Outside of this region, the extent of similarity was lower. The plant RNA contains an Sm protein binding site located at a similar position with a single T-to-A deviation from the consensus sequence (Fig. 6). Most of the size differ-

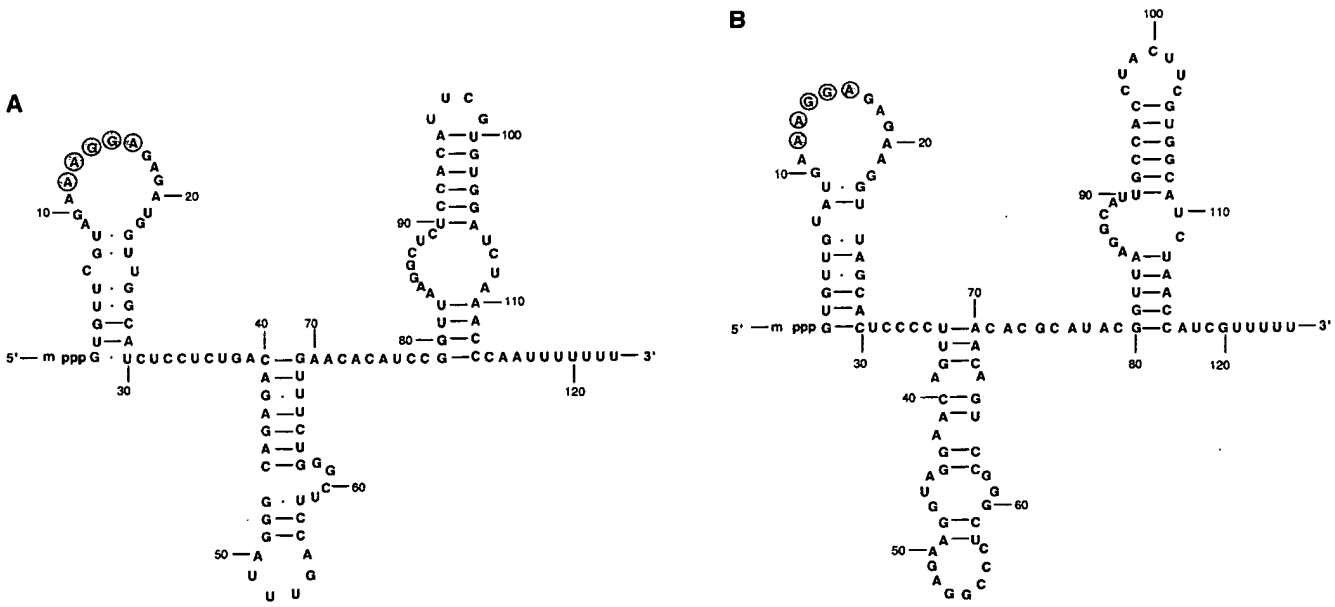

FIGURE 3. Proposed secondary structures of U6atac snRNAs. The nucleotides that can base pair to the intron 5′ splice site are circled. **A**: *Arabidopsis* U6atac snRNA. **B**: Human U6atac snRNA (adapted from Tarn & Steitz, 1996b).

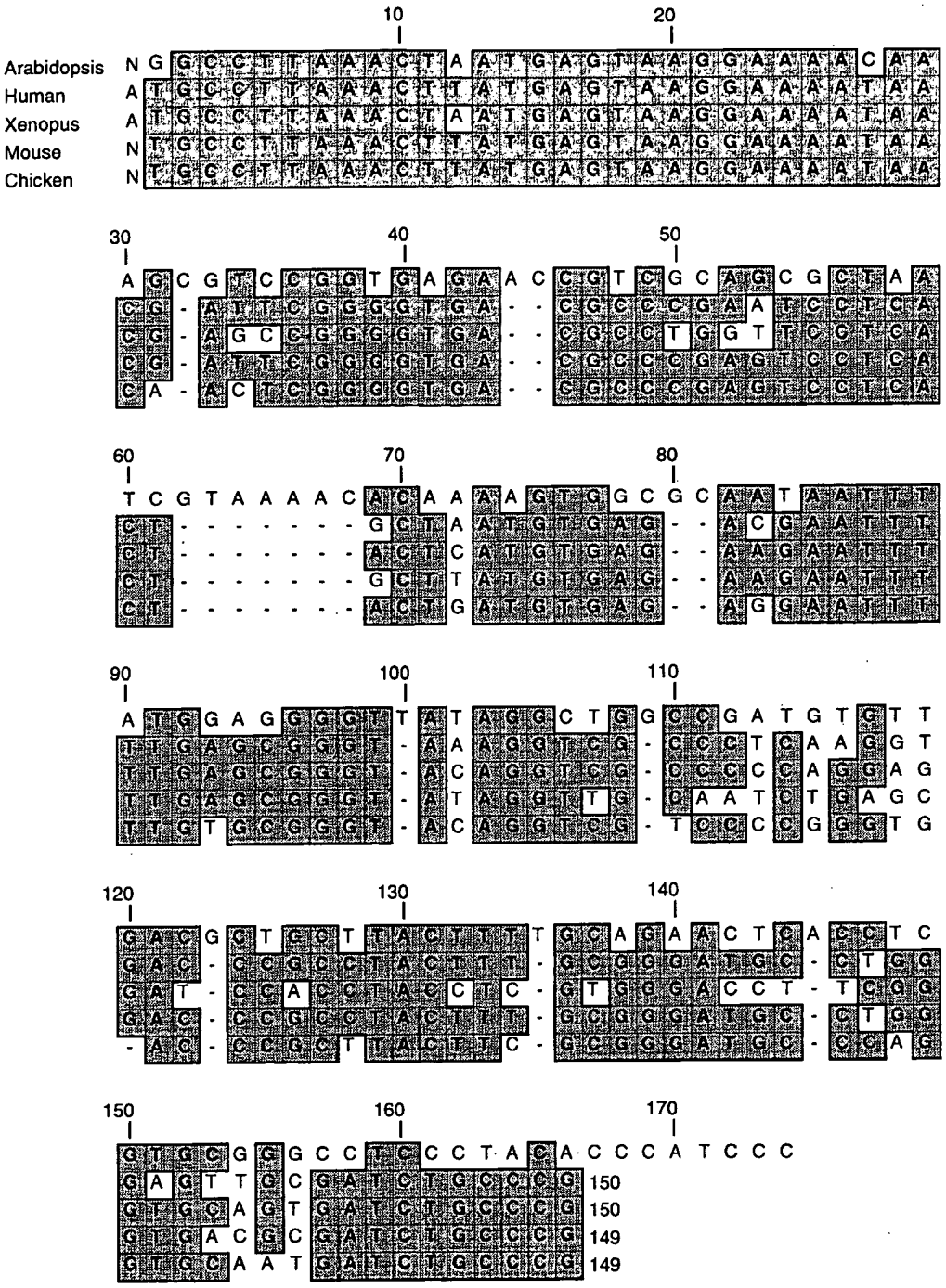

FIGURE 4. Sequence comparison of the putative *Arabidopsis* U12 snRNA with human, chicken, mouse, and *Xenopus* U12 snRNAs (Tarn et al., 1995; Yu et al., 1996). The nucleotide numbering is for the *Arabidopsis* sequence. Conserved nucleotides are boxed and gaps introduced into the alignment are shown as hyphens (-).

ence between the plant and human U12 snRNAs is because of an additional sequence of seven internal and eight 3′ nucleotides in the plant RNA.

This putative plant U12 snRNA homolog can be folded into a hypothetical secondary structure very similar to that determined for human U12 snRNA (Montzka Wassarman & Steitz, 1992; Fig. 6). In this structure, the 7-nt additional internal sequence in the plant homolog is placed in the loop region of stem-loop III. In addition, many of the differences between the plant and human sequences appear to be due to compensatory changes that maintain base pairs in the stem regions.

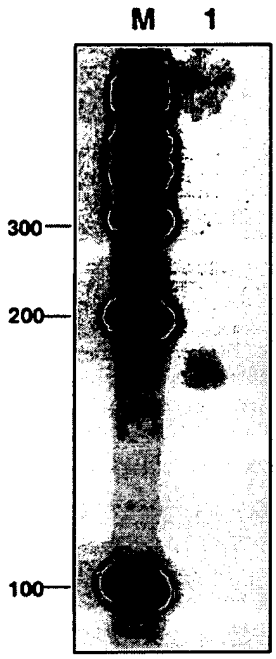

FIGURE 5. Expression of the *Arabidopsis* U12 snRNA shown by Northern blot analysis of total RNA. Ten micrograms of total RNA from *Arabidopsis* was fractionated on a 8% denaturing polyacrylamide gel and transferred to a nylon membrane. The blot was hybridized with ^{32}P-labeled U12 snRNA cDNA. The sizes of the RNA markers in nucleotides are given at the left.

Conservation of proposed interactions in the U12-dependent spliceosome

A major goal of this work was to use phylogenetic comparisons between the plant and human snRNA sequences to evaluate the RNA–RNA interactions proposed for the U12-dependent spliceosome. Tarn and Steitz (1996b) have proposed a model of the RNA interactions between U12 and U6atac based on analogy with the U2-U6 snRNA interactions and the demonstration of a U12-U6atac crosslink. Figure 7 shows a modified version of this model that, in addition to the U12-U6atac pairing, also shows the base pairing of U12 snRNA to the intron branch site sequence and the pairing of U11 and U6atac snRNAs to the intron 5′ splice site sequence as established in mammalian U12-dependent splicing (Hall & Padgett, 1996; Tarn & Steitz, 1996a, 1996b; Yu & Steitz, 1997; Incorvaia & Padgett, 1998). The nucleotides that differ from the human sequences are shown in bold type. The analogous human structure is shown in Figure 8A. Since the regions of U12 and U6atac snRNAs that are involved in these potential interactions are the most conserved regions of both snRNAs, it is not surprising that the human and plant structures would be very similar. As expected from the conservation of the intronic splice site sequences, the regions of plant U12 and U6atac snRNAs that interact with the splice sites are identical to the human sequences.

Also identical in plant U6atac snRNA is the AGAGA sequence immediately following the 5′ splice site pairing region. This sequence is also completely conserved in U6 snRNAs (Brow & Guthrie, 1988) and is required for function in yeast U6 snRNA (Fabrizio & Abelson, 1990; Madhani et al., 1990). Immediately following this sequence is the region believed to base pair to U12 snRNA to form the two-part helix I interaction (Tarn & Steitz, 1996b). The plant U6atac snRNA differs from the human sequence in two positions in this region. There is a U in place of an A at position 21 that appears to be compensated for by an A-to-U change in U12 snRNA. These changes maintain the potential A-U base pair as shown. The second difference is an A-to-G change in helix Ib at position 26. This alters the AGC sequence in this region to GGC and allows an additional G-C base pair to be made to U12 snRNA. This would also have the effect of enlarging the bulge in U12 snRNA between helix Ia and helix Ib to 3 nt. In U6 snRNA, alterations to the highly conserved AGC sequence at the analogous position have severe effects on splicing, with mutations of the A residue leading to a block at the second step of splicing in yeast (Fabrizio & Abelson, 1990; Madhani et al., 1990). The role of this change in U6atac snRNA is addressed below.

Immediately following this AGC in human U6atac snRNA is an intramolecular stem-loop structure which extends helix Ib. This stem-loop is very similar in size and structure to a conserved stem-loop structure located at the analogous position in U6 snRNA. The plant U6atac homolog conserves the structural features of this stem-loop but has altered bases at 9 of the 21 positions compared to human U6atac. Investigations of this structure in human U6 snRNA indicate that, although the base pairs are important for function, the identities of the bases are not (Sun & Manley, 1997). As is shown in Figure 7, nine of the ten changes can be accommodated in the same base-paired structure as in the human homolog (Fig. 8A). The sole exception is the change of the bulged U 46 residue to a C. The structure shown in Figure 7 is slightly different from the original proposal for this region of human U6atac snRNA (Tarn & Steitz, 1996b). The pattern of compensatory base changes between the human and plant sequences suggests that both A 45 and C (plant) or U (human) 46 are bulged rather than having a single bulged residue at position 46. This change does not alter the upper part of the stem, but does rearrange the base pairs in the lower section, resulting in fewer noncanonical base pairs, and increases the calculated stabilities of both structures by 2–3 kcal/mol. The actual structure that this region adopts in the spliceosome is likely to be influenced by additional tertiary RNA interactions and interactions with proteins.

As in the case of human U12 and U6atac snRNAs (Tarn & Steitz, 1996b), an interaction analogous to the U2-U6 helix II (Datta & Weiner, 1991) cannot be made.

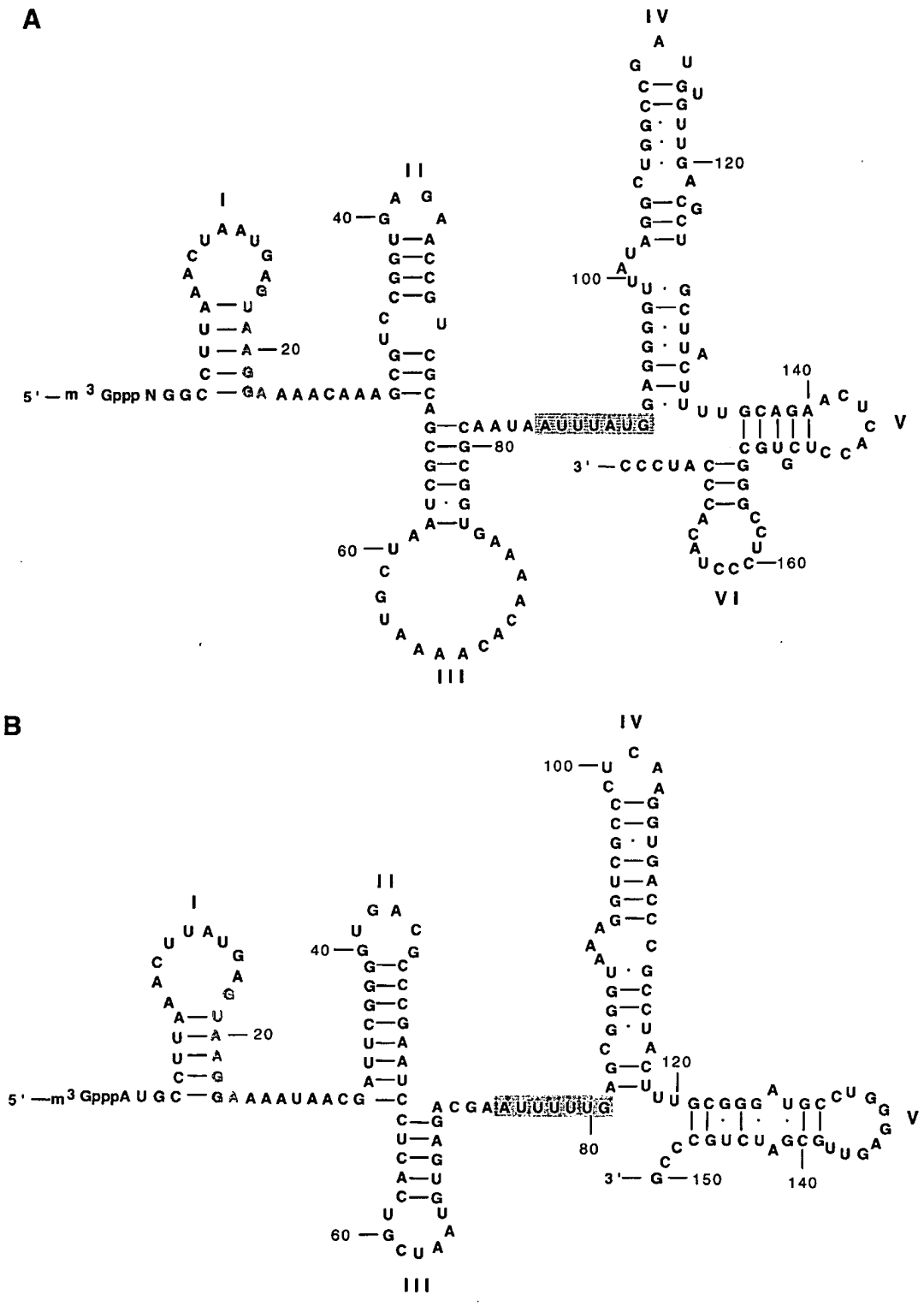

FIGURE 6. Proposed secondary structures of U12 snRNAs. The nucleotides that can base pair to the intron branch site are in outline type. The probable Sm protein binding sites are shaded. **A**: *Arabidopsis* U12 snRNA. **B**: Human U12 snRNA (adapted from Tarn et al., 1995).

In addition, the potential for base pairs between U12 and U6atac near the 5′ end of U6atac (Tarn & Steitz, 1996b) that resemble the U2-U6 helix III (Sun & Manley, 1995) is not conserved in the plant homologs (compare Figs. 7 and 8A).

The U6atac snRNA intramolecular stem-loop structure can be functionally transplanted

One of the intriguing conservations observed when comparing U6 and U6atac snRNAs is in the structure of the

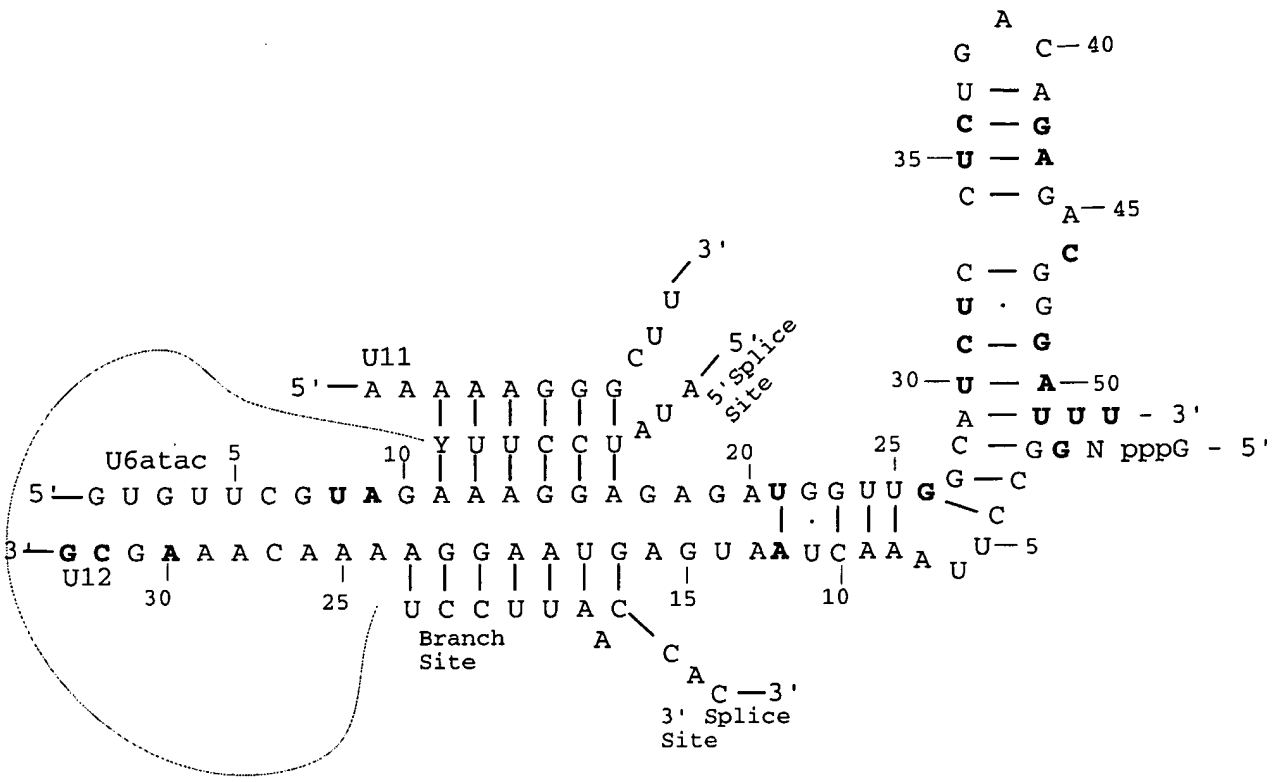

FIGURE 7. Diagram of the RNA–RNA interactions proposed in the *Arabidopsis* U12-dependent spliceosome. The potential inter- and intramolecular base-pairing interactions of the plant U12 and U6atac snRNAs are diagramed. The differences from the human homologs of U12 and U6atac snRNAs are indicated by bold characters. The human U11 snRNA sequence is shown for illustration only, as we have not determined the plant U11 snRNA sequence.

intramolecular stem-loop that immediately follows the region of interaction with U2 or U12 respectively. This feature of U6 snRNA is required for splicing, but appears to be tolerant of mutations that maintain the structure (Sun & Manley, 1997). As noted above, the plant and human U6atac snRNAs differ at 9 positions out of 21 within this region yet have a very similar predicted structure. We wanted to test the hypothesis that both stem-loops were also functionally homologous. To do this, we introduced all nine mutations into the stem-loop region of a previously described human U6atac suppressor snRNA expression construct (Incorvaia & Padgett, 1998). The parent construct contains a double mutation in the region that base pairs with the 5′ splice site that suppresses a 5′ splice site mutation when coexpressed with a similarly altered U11 snRNA construct (Incorvaia & Padgett, 1998). This suppression assay allows us to determine the in vivo functional effects of second site mutations of U6atac in the presence of the endogenous wild-type U6atac snRNA.

The specific mutations are shown in Figure 8A (except for the A26G mutation which was tested separately; see below) and the results of the in vivo splicing suppression assay are shown in Figure 9. The results show that, as we previously demonstrated (Incorvaia & Padgett, 1998), the cryptic splicing phenotype of the P120 CC5/6GG 5′ splice site mutant is suppressed to yield correctly spliced mRNA when U11 and U6atac snRNAs containing compensatory mutations in their 5′ splice site interaction regions are coexpressed (compare Fig. 9, lanes 4 and 9). However, when the human U6atac stem-loop is replaced by the plant stem-loop in this construct, no suppression is observed (Fig. 9, lane 11) suggesting that this chimeric U6atac snRNA is nonfunctional.

This result could be due to any of several reasons. For example, the chimeric U6atac snRNA might be unstable, it might not interact productively with human U4atac snRNA, or it might not interact with the other elements of the human U12-dependent spliceosome. Of these possibilities, a problem with the interaction with U4atac could be tested in vivo. As shown in Figure 8B, the region of U6atac snRNA that encompasses the intramolecular stem-loop is also a region that base pairs extensively with U4atac snRNA. The mutations introduced by transplanting the plant stem-loop sequence significantly reduced the base pairing potential of the chimeric U6atac with wild-type U4atac snRNA.

To compensate for this, we prepared an expression construct for U4atac snRNA as we had for U11 and U12 snRNAs (Hall & Padgett, 1996; Kolossova & Padgett, 1997) by replacing the snRNA coding region

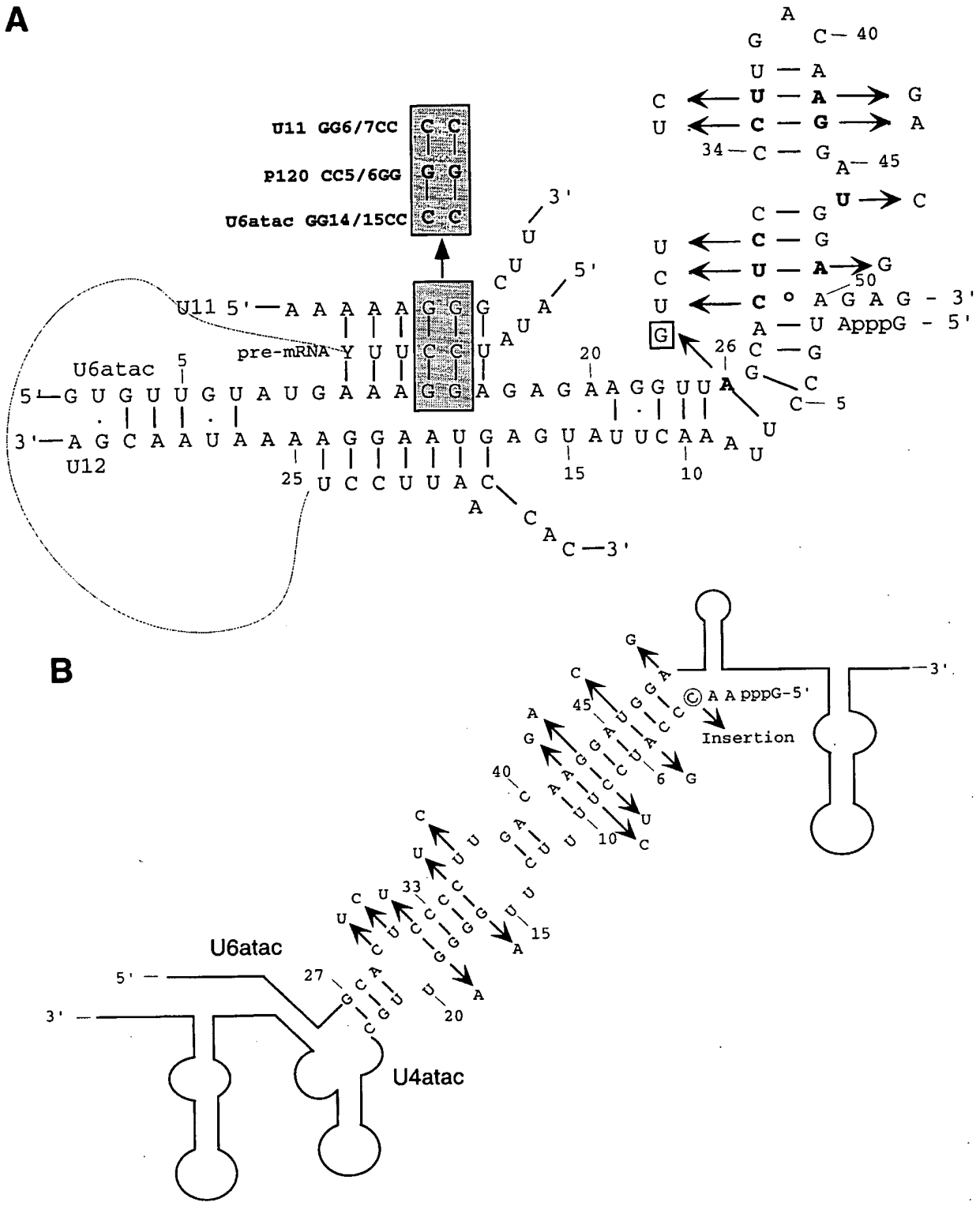

FIGURE 8. A: Mutational analysis of U6atac snRNA function. The P120 5′ splice site mutation CC5/6GG is shown above the figure with the compensating mutations in U11 and U6atac snRNAs. The base alterations tested in the in vivo mutant suppressor assay are shown. The chimeric U6atac snRNA construct contained the mutations shown from C30 through A49. The A26G mutation (boxed) was tested separately. **B**: Diagram of the proposed base-pairing interaction between human U4atac and U6atac. The arrows on the U6atac sequence show the changes that were made in this region by constructing the chimeric U6atac snRNA using the *Arabidopsis* intramolecular stem-loop structure. The arrows on the U4atac sequence show the changes made in the human U4atac expression construct to compensate for the lost base pairs in the U6atac chimera.

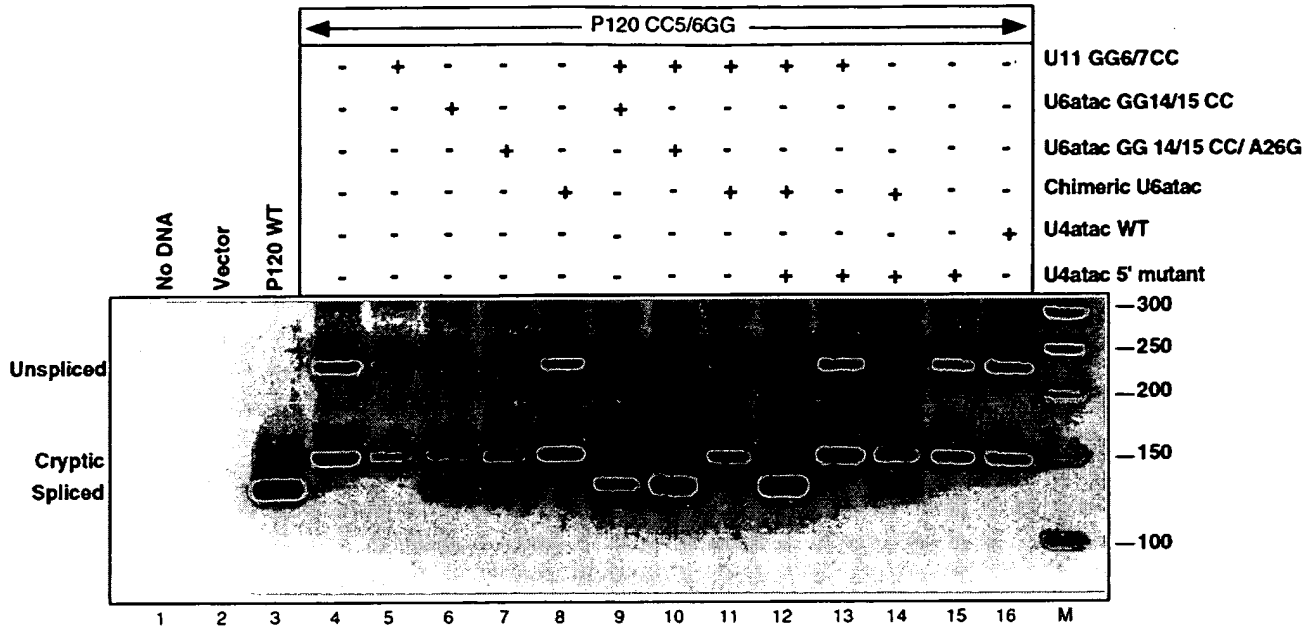

FIGURE 9. In vivo functional analysis of mutant and chimeric U6atac snRNAs. The indicated P120 minigene constructs were transfected into CHO cells along with expression constructs for the various snRNAs. RNA extracted after 48 h was analyzed by RT-PCR for the splicing pattern of the P120 minigene. The three major products correspond to unspliced intron F RNA (Unspliced), RNA spliced via the U2-dependent spliceosome using internal 5′ and 3′ cryptic splice sites (Cryptic) and correctly spliced P120 RNA (Spliced). Lane 1 is mock transfected CHO cell RNA. Lane 2 is RNA from cells transfected with the pCB6 expression vector. Lane 3 is RNA from cells transfected with the wild-type P120 expression construct. Lanes 4–16 are RNA samples from cells transfected with the intron F 5′ splice site mutant CC5/6GG and the indicated snRNA construct(s).

of a functional human U1 snRNA gene with the U4atac snRNA sequence. In addition, we prepared a mutant U4atac snRNA expression construct that contained compensating mutations to restore base pairing to the chimeric U6atac snRNA. We then tested the combination of the mutant U4atac with the chimeric U6atac snRNAs in the in vivo splicing suppression assay. As shown in Figure 9, the mutant U4atac could activate the suppression activity of the chimeric U6atac snRNA (lane 12). The mutant U4atac by itself (Fig. 9, lane 15) or with the suppressor U11 snRNA (Fig. 9, lane 13) was inactive for in vivo suppression.

These results show (1) that the U6atac intramolecular stem-loop can retain function in spite of the alteration of close to half of its residues; (2) that its function in splicing is conserved between plants and humans; (3) that this region of U6atac must also participate in base pairing interactions with U4atac snRNA for expression and/or function; and (4) that U4atac plays an essential role in U12-dependent pre-mRNA splicing through its interaction with U6atac snRNA.

The lack of conservation of A 26 does not affect function of U6atac

As noted above, the plant U6atac snRNA differs from human U6atac at position 26 in the helix Ib region. The sequence of the analogous region of U6 snRNA contains a highly conserved AGC motif which is also found in human U6atac snRNA. The homologous sequence in plant U6atac snRNA is GGC. Mutation of this region in yeast U6 snRNA leads to defects in splicing (Fabrizio & Abelson, 1990; Madhani et al., 1990). In particular, mutants of the A residue show a phenotype consistent with a second step defect. In light of this, the possibility existed that the plant U6atac sequence that we identified could be an inactive isoform although we had no evidence for a second species.

To rule out this possibility, we tested the effect of the A 26 G mutation in human U6atac snRNA using the same method as above. We constructed a U6atac snRNA expression vector containing the GG14/15CC mutation and the A 26 G mutation and tested it for activity in suppression of the P120 5′ splice site CC5/6GG mutant. As shown in Figure 9, lanes 7 and 10, the position 26 mutation had no effect on the suppression activity of U6atac snRNA. These results show that this mutation at position 26 is fully compatible with U12-dependent splicing in vivo.

DISCUSSION

From the time of their first identification, the virtually complete conservation of the splice site and branch

site sequences of U12-dependent introns has been a distinctive feature of this group of introns (Hall & Padgett, 1996). The evolutionary distance over which this conservation holds was significantly extended by the recognition of U12-dependent introns in plants (Wu et al., 1996). Subsequently, many more U12-dependent introns have been identified in plant genomic sequences (Burge et al., 1998a). The splice site sequences of all of these plant introns fall in the same range of consensus scores as animal U12-dependent introns suggesting that there are no plant-specific modifications of the consensus. This, in turn, suggested that the snRNAs that recognize these splice site sequences would likely show similar conservation of the interacting regions. This conservation should allow us to identify the plant homologs of the human U12-dependent spliceosomal snRNAs. We therefore used database searches and biochemical approaches to identify the plant U12 and U6atac snRNA homologs based on the conservation of the sequences that interact with splice sites.

The putative U6atac snRNA homolog that we identified in *Arabidopsis* is likely to be the authentic U6atac snRNA based on several lines of evidence. We have shown that the sequence we identified in the genome database is expressed in plant cells as a small RNA with ends corresponding to those predicted by comparison to human U6atac snRNA. In addition, this gene sequence contains all the elements known to be required to promote proper transcription of U6-like genes in plants (Waibel & Filipowicz, 1990). The sequence of the expressed RNA shows complete conservation of the 5′ splice site interacting region in agreement with the conservation of the 5′ splice site sequence between plants and humans (Burge et al., 1998a). There is almost complete identity between the plant and human sequence in the region between the splice site pairing region and the intramolecular stem-loop. This region encompasses both the AGAGA sequence, which is invariant in U6 snRNA (Brow & Guthrie, 1988) and also found in human U6atac snRNA (Tarn & Steitz, 1996b), as well as the region of potential base pairing to U12 snRNA. Of the 2 nt that differ in this region between plants and humans, the A-to-U change at position 21 appears to be compensated by a U-to-A change at position 14 of U12 snRNA, thus maintaining the base pair at this position. Interestingly, this same change is seen in *Xenopus* U12 snRNA (Yu et al., 1996). Whether a similar compensatory change exists in *Xenopus* U6atac snRNA is not known. The second change in *Arabidopsis* U6atac snRNA of G for A at position 26 does not appear to affect U6atac function (see below). Finally, the intramolecular stem-loop that immediately follows this region is only about 50% conserved in sequence but can be folded into a similar structure and is fully functional when transplanted into human U6atac snRNA (see below). All of these findings support the identification of this RNA as the plant U6atac snRNA homolog.

A similar set of arguments applies to the putative plant U12 snRNA homolog identified here. The sequence is expressed as a small RNA in plants. The size and predicted structure are similar to those of vertebrate U12 snRNAs. The region that is predicted to base pair to the intron branch site sequence is identical to human, as is the branch site sequence itself. The region predicted to base pair to U6atac snRNA is also very highly conserved, with only one compensatory base change that maintains pairing to the plant U6atac homolog. Finally, this RNA appears to be the only plant small RNA that contains these conserved elements. Both Northern and RACE analyses failed to detect other RNAs using the 5′ end of human U12 snRNA. A recent addition to the *Arabidopsis* genome database contained a sequence (Accession #AC004255) with high similarity to U12 snRNA of human and even greater similarity to the expressed plant sequence that we identified. The database sequence contains several differences from the plant RNA we describe here. None of these differences have been seen in the 31 cDNA clones we have sequenced. This suggests that this sequence is not expressed at an appreciable level in *Arabidopsis* and thus it most likely corresponds to a U12 snRNA pseudogene.

Functional testing of nonconserved elements of U6atac snRNA

To determine if some of the differences noted above between plant and human U6atac snRNAs were functionally silent, we tested them in the context of an in vivo suppression assay for U6atac snRNA. We have previously shown that human U6atac snRNA compensatory mutants expressed in Chinese hamster ovary (CHO) cells can suppress the in vivo cryptic splicing phenotype of 5′ splice site mutants of a U12-dependent intron (Incorvaia & Padgett, 1998). Starting with this suppressor snRNA, we tested the effect of the A-to-G difference at position 26 of plant U6atac. This position appears to be homologous to the invariant A of the AGC motif found in U6 snRNAs (Brow & Guthrie, 1988) and in human U6atac snRNAs (Tarn & Steitz, 1996b). In yeast U6 snRNA, mutation of this position leads to defects in splicing, particularly in the second step (Fabrizio & Abelson, 1990; Madhani et al., 1990). However, mutation of this residue in mammalian U6 snRNA had no effect using an in vivo suppression assay (Datta & Weiner, 1993). When the A 26-to-G mutation was introduced into the suppressor U6atac construct (Figs. 8A and 9), full in vivo suppressor activity was maintained showing that G 26 is fully compatible with active U12-dependent splicing. Note that both A 26 and G 26 can potentially base pair to U12 snRNA in slightly different registers (Figs. 7 and 8A).

Immediately following this (A/G)GC sequence is a region that can form an intramolecular stem-loop that is similar in size, position, and structure to a critical region

of U6 snRNA. Although the plant and human U6atac sequences differ by almost 50% in this region, the predicted structures are similar. To demonstrate that the plant stem-loop could still be active in spite of these differences, we constructed a chimeric U6atac in which the plant stem-loop replaced the human stem-loop. The resulting construct was tested for activity in vivo using the same suppressor assay described above. We found that, in the presence of a mutated human U4atac snRNA, this chimeric U6atac snRNA was active in vivo (Figs. 8A and 9). This shows that the function of the plant intramolecular stem-loop structure is conserved.

These data also provide the first in vivo evidence for the predicted function of U4atac snRNA in U12-dependent splicing. When Tarn and Steitz (1996b) identified U6atac and U4atac snRNAs in human nuclear extracts, they noted that the two snRNAs could adopt a base-paired structure analogous to that formed by U4 and U6 snRNAs. In the case of U4/U6 snRNA, this structure appears to be required for splicing in vivo and in vitro (Wolff & Bindereif, 1992). The precise role of this structure is still unclear but it has been proposed that U4 snRNA acts as a chaperone to deliver the U6 snRNA to the nascent spliceosome in an inactive form (Guthrie & Patterson, 1988). Subsequently, through the action of ATP-dependent helicases (Raghunathan & Guthrie, 1998), the two snRNAs are separated and U6 goes on to form alternative base-pairing interactions with U2 and the intron 5′ splice site whereas U4 appears to be destabilized from the spliceosome (Lamond et al., 1988; Yean & Lin, 1991; reviewed in Nilsen, 1998).

The provocative potential sequence complementarities between U4atac and U6atac, in addition to their copurification, led to the proposal that they participated in an analogous interaction (Tarn & Steitz, 1996b). Our initial experiments with the chimeric human/plant U6atac snRNA showed that it was inactive in the in vivo suppression assay. Inspection of the sequence showed that the altered region of the chimeric snRNA also corresponded to the region proposed to form many of the base pairs to U4atac snRNA. The plant sequence differs substantially in this region and would be expected to destabilize the interaction with U4atac. To test if this was the cause of the failure of the chimeric U6atac snRNA to suppress, we constructed a human U4atac snRNA expression gene and made compensatory mutations in the 5′ region to restore the same base-paired structure as in human U4atac and U6atac snRNAs (Fig. 8B). Cotransfection of this compensatory U4atac expression construct led to suppression of the intron 5′ splice site mutation in a manner that required both the chimeric U6atac and the compensatory U4atac constructs (Fig. 9). This demonstrates that there is an in vivo requirement for base pairing between U4atac and U6atac snRNAs.

Conserved features of plant U6atac and U12 snRNAs

The evolutionary distance between plants and vertebrates has permitted numerous changes to accumulate in their respective snRNAs. This is particularly noteworthy in the case of U6atac snRNA. The *Arabidopsis* and human homologs of this snRNA are significantly more divergent than are U6 snRNAs from the same species (65% for U6atac versus 85% for U6). The source of this difference is unknown at present. A possibility is that there are only one or a few active genes for U6atac. It has been reported that the human U12 snRNA gene is single copy (Tarn et al., 1995). The precise number of U6atac genes that exist in any organism is unknown at present, but preliminary Southern analyses of *Arabidopsis* genomic DNA suggest that there are only a small number of U6atac genes (data not shown). If these snRNAs are expressed from single-copy genes, it would reduce the potential for sequence "homogenization" seen in multigene families and so speed the accumulation of functionally silent changes.

The pattern of conservation of U6atac sequences corresponds well to the predicted functional regions of the molecule. Of particular note is the conservation of sequence and the potential for base pairing in the U6atac-U12 helix Ia/Ib regions. Biochemical crosslinking experiments suggested that these regions were juxtaposed (Tarn & Steitz, 1996b). The conservation of this complementarity in plants, including a clear case of a base pair in helix Ia being preserved through compensatory base alterations, strengthens this view. The conservation of the nominally unpaired bases in U12 snRNA in this region suggests that they may have additional roles in the U12-dependent spliceosome. Similarly, the sequence differences seen in the intramolecular stem-loop of U6atac are restricted almost entirely to nucleotides which appear to be engaged in base-pairing interactions. With one exception, all of the putative unpaired bases are conserved between plants and humans. A strong but not complete correspondence of these bases between U6atac and U6 snRNAs has been noted (Tarn & Steitz, 1997). Whether this similarity is because of a need to interact with common proteins or to form a catalytic RNA structure or both is unclear at present. In contrast, the potential to form a U6atac-U12 interaction analogous to the U2-U6 helix III (Sun & Manley, 1995) is not conserved in the plant homologs. Both snRNAs differ from the human sequences in this region, with no clear evidence of compensatory changes suggestive of a base-pairing interaction.

MATERIALS AND METHODS

RT-PCR cloning of *Arabidopsis* U6atac snRNA

A candidate *Arabidopsis* U6atac snRNA gene was identified by a BLAST search of available sequences using the first

40 nt of human U6atac snRNA. This identified a single high-scoring entry (Accession #AB006702). To determine if this putative snRNA was expressed, first-strand cDNA was reverse transcribed from 0.5 μg of total *Arabidopsis* RNA using Tth polymerase (Perkin-Elmer). The reaction was primed with the antisense primer (CACGAATGTGGAGAGCCTTAAC) spanning the region between 80 and 101 of the putative plant U6atac snRNA gene sequence at 60 °C for 15 min. For amplification of the U6atac cDNA, a sense primer (GTGTTCG TAGAAAGGAGAGATGG) spanning bases 1–23 of the putative U6atac snRNA was used together with the antisense primer above. The cDNA was amplified for 1 min at 95 °C, 1 min at 60 °C, and 1 min at 72 °C for 40 cycles followed by 7 min at 72 °C.

RT-PCR of *Arabidopsis* U12 snRNA

Total *Arabidopsis* RNA was fractionated on a 10% denaturing polyacrylamide gel and RNAs between 100 and 200 nt were isolated by the "Crush-Soak" procedure (Sambrook et al., 1989). The eluted RNA was extracted with phenol and chloroform, ethanol precipitated, washed, and dissolved in water. The RNA (0.2 μg) was 3′ polyadenylated using yeast poly (A) polymerase (Gibco/BRL) and 2 mM ATP in the supplied buffer for 10 min at 37 °C in a total volume of 25 μL. First-strand cDNA was synthesized from 1/10 of the poly (A)-tailed RNA by reverse transcription using an oligo d(T) primer and SuperScript reverse transcriptase in the supplied buffer and 0.5 mM of each dNTP at 42 °C for 1 h in a 20-μL reaction. The U12 cDNA was amplified using a primer (ATGCCTTAAACTTATGAGTAAGGA) derived from human U12 snRNA spanning bases 1 to 24. Amplification parameters were 1 min at 95 °C, 1 min at 56 °C, and 1 min at 72 °C for 40 cycles followed by 7 min at 72 °C.

Characterizing the ends of U6atac and U12 snRNAs by RACE

For U12 snRNA, the 3′ end sequence was determined from amplification of poly (A)-tailed RNA using oligo dT and a human U12-specific 5′ primer. Twenty-five clones of this product were sequenced. The true 5′ end sequence was determined using the SMART PCR cDNA kit (Clontech) to attach a primer to the 3′ end of the cDNA, followed by PCR amplification using a primer complementary to this sequence and an internal U12-specific primer. The amplified DNA was gel purified, cloned, and sequenced.

For U6atac snRNA, the 3′ end sequence was determined by amplifying the poly (A)-tailed RNA using the 5′ U6atac primer (nt 1–23) and a modified oligo dT primer (CDS/3′ primer, Clontech). The amplified DNA was gel purified, cloned, and sequenced. The 5′ end sequence was determined from cDNA synthesized from the poly (A)-tailed RNA using the CDS/3′ primer that was 3′ tailed using dCTP and terminal deoxynucleotidyl transferase. The cDNA was amplified with the abridged anchor primer above and a primer complementary to nt 80–101 of U6atac snRNA. The amplified DNA was then gel purified, cloned, and sequenced.

Construction of U4atac expression plasmid

The U4atac expression plasmid was generated by the same method used previously for U11 and U12 snRNAs. Briefly, the U1 snRNA coding region of a functional U1 gene was replaced by PCR techniques with the coding region of U4atac snRNA amplified from a U4atac plasmid obtained from J. Steitz. Sequence analysis of this plasmid showed that it was missing the 3′-most 7 nt of the published U4atac sequence and contained a sequence alteration at residues 60 and 61. This sequence alteration leads to a CG-to-GC inversion at these residues when compared to the published sequence (Tarn & Steitz, 1996b) and appears to represent an error in the original sequence determination (J. Steitz, pers. comm.). For the expression plasmid described here, the final 7 nt were supplied by the PCR primer, but the inversion at nt 60–61 was left unchanged. The wild-type U4atac primers were GGCCAGATCTCAACCATCCT TTTCTTGGGGT (5′ primer) and (3′ primer) CCGG GTCGACGGTCTGTTTTTGAAACTCCAGAAAGTCTATTTTT CCAAAAATTGCAC. The mutant 5′ primer was GGCC AGATCTCAACCCGTCTCTTTCTTAGGATTGCGCTACTGTC. These produced PCR products containing either human wild-type U4atac or human U4atac containing compensatory mutations to restore base pairing with the chimeric U6atac snRNA. The PCR fragments were digested with *Sal*I and *Bgl*II restriction enzymes and ligated into a U1 expression vector from which the U1 coding region had been excised (Bond et al., 1991). The sequences of the mutant and wild-type snRNAs were confirmed by DNA sequencing.

Construction of U6atac mutants

The U6atac snRNA mutants were made in the expression plasmid previously described (Incorvaia & Padgett, 1998) using the Altered Sites II system from Promega and single mutagenic oligonucleotides. The sequence of the primer used for synthesis of the U6atac chimeric snRNA construct was GAAGGTTAGCATCTCCTCTGACAGAGACGGGAGAGGC CCTC. All mutations were confirmed by DNA sequencing.

Analysis of in vivo splicing

Transient transfection of the P120 minigene and snRNA expression plasmids into cultured CHO cells was as described (Hall & Padgett, 1996; Kolossova & Padgett, 1997; Incorvaia & Padgett, 1998). For these experiments, 0.5 μg of P120 plasmid and 5 μg of each of the snRNA expression plasmids were added to 1×10^6 cells. Where one or more snRNA plasmids were omitted, a corresponding amount of pUC19 plasmid DNA was substituted. Total RNA was isolated from cells 48 h after transfection, reverse transcribed, and PCR amplified as described (Kolossova & Padgett, 1997; Incorvaia & Padgett, 1998). The products were analyzed by agarose gel electrophoresis. The DNA bands were visualized using ethidium bromide and photographed using a digital video camera (Kodak). Independent transfections and analyses gave substantially similar results.

Northern blot analysis

Ten micrograms of total cellular *Arabidopsis* RNA was fractionated on a 8% polyacrylamide-urea gel and electroblotted to a nylon membrane. Prehybridization was carried out at 60°C for 2 h in 6× SSC, 5× Denhardt's solution, 0.1% SDS,

and 0.1 mg/mL of sonicated salmon sperm DNA. Probes were prepared by random priming of cDNA plasmids using ^{32}P-dCTP and hybridized to the blots in the above solution at a concentration of 10^4 cpm/mL. Hybridizations were performed at 60 °C for 16 h. The membranes were washed at room temperature for 15 min in 2× SSC and then at 60 °C in 2× SSC and 0.05% SDS for 20 min.

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REFERENCES

Bond UM, Yario TA, Steitz JA. 1991. Multiple processing-defective mutations in a mammalian histone pre-mRNA are suppressed by compensatory changes in U7 RNA both in vivo and in vitro. *Genes & Dev 5*:1709–1722.

Brow DA, Guthrie C. 1988. Spliceosomal RNA U6 is remarkably conserved from yeast to mammals. *Nature 334*:213–218.

Brown JWS, Simpson CG. 1998. Splice site selection in plant pre-mRNA splicing. *Annu Rev Plant Physiol Plant Mol Biol 49*:77–95.

Burge CB, Padgett RA, Sharp PA. 1998a. Evolutionary fates and origins of U12-type introns. *Mol Cell 2*:773–785.

Burge CB, Tuschl T, Sharp PA. 1998b. Splicing of precursors to mRNAs by the spliceosome. In: Gestland RF, Cech T, Atkins JF, eds. *The RNA world II*. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press. pp 525–560.

Datta B, Weiner AM. 1991. Genetic evidence for base pairing between U2 and U6 snRNA in mammalian mRNA splicing. *Nature 352*:821–824.

Datta B, Weiner AM. 1993. The phylogenetically invariant ACAGAGA and AGC sequences of U6 small nuclear RNA are more tolerant of mutation in human cells than in *Saccharomyces cerevisiae*. *Mol Cell Biol 13*:5377–5382.

Fabrizio P, Abelson J. 1990. Two domains of yeast U6 small nuclear RNA required for both steps of nuclear precursor messenger RNA splicing. *Science 250*:404–409.

Guthrie C, Patterson B. 1988. Spliceosomal snRNAs. *Annu Rev Genet 22*:387–419.

Hall SL, Padgett RA. 1994. Conserved sequences in a class of rare eukaryotic introns with non-consensus splice sites. *J Mol Biol 239*:357–365.

Hall SL, Padgett RA. 1996. Requirement of U12 snRNA for the in vivo splicing of a minor class of eukaryotic nuclear pre-mRNA introns. *Science 271*:1716–1718.

Incorvaia R, Padgett RA. 1998. Base pairing with U6atac snRNA is required for 5′ splice site activation of U12-dependent introns in vivo. *RNA 4*:709–718.

Jackson IJ. 1991. A reappraisal of non-consensus mRNA splice sites. *Nucleic Acids Res 19*:3795–3798.

Kolossova I, Padgett RA. 1997. U11 snRNA interacts in vivo with the 5′ splice site of U12-dependent (AU-AC) introns. *RNA 3*:227–233.

Lamond AI, Konarska MM, Grabowski PJ, Sharp PA. 1988. Spliceosome assembly involves the binding and release of U4 small nuclear ribonucleoprotein. *Proc Natl Acad Sci USA 85*:411–415.

Madhani HD, Bordonne R, Guthrie C. 1990. Multiple roles for U6 snRNA in the splicing pathway. *Genes & Dev 4*:2264–2277.

Montzka Wassarman K, Steitz JA. 1992. The low-abundance U11 and U12 small nuclear ribonucleoproteins (snRNPs) interact to form a two-snRNP complex. *Mol Cell Biol 12*:1276–1285.

Nilsen TW. 1998. RNA–RNA interactions in nuclear pre-mRNA splicing. In: Simons RW, Grunberg-Manago M, eds. *RNA structure and function*. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press. pp 279–307.

Raghunathan PL, Guthrie C. 1998. RNA unwinding in U4/U6 snRNPs requires ATP hydrolysis and the DEIH-box splicing factor Brr2. *Curr Biol 8*:847–855.

Reddy R, Busch H. 1988. Small nuclear RNAs: RNA sequences, structure, and modifications. In: Birnstiel ML, ed. *Structure and function of the major and minor small nuclear ribonucleoprotein particles*. Berlin: Springer-Verlag. pp 1–37.

Sambrook J, Fritsch EF, Maniatis T. 1989. *Molecular cloning: A laboratory manual*. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press.

Simpson GG, Filipowicz W. 1996. Splicing of precursors to mRNA in higher plants: Mechanism, regulation and sub-nuclear organization of the spliceosomal machinery. *Plant Mol Biol 32*:1–41.

Sun J-S, Manley JL. 1995. A novel U2-U6 snRNA structure is necessary for mammalian mRNA splicing. *Genes & Dev 9*:843–854.

Sun J-S, Manley JL. 1997. The human U6 snRNA intramolecular helix: Structural constraints and lack of sequence specificity. *RNA 3*:514–526.

Tarn W-Y, Steitz JA. 1996a. A novel spliceosome containing U11, U12, and U5 snRNPs excises a minor class (AT-AC) intron in vitro. *Cell 84*:801–811.

Tarn W-Y, Steitz JA. 1996b. Highly diverged U4 and U6 small nuclear RNAs required for splicing rare AT-AC introns. *Science 273*:1824–1832.

Tarn W-Y, Steitz JA. 1997. Pre-mRNA splicing: The discovery of a new spliceosome doubles the challenge. *Trends Biochem Sci 22*:132–137.

Tarn W-Y, Yario TA, Steitz JA. 1995. U12 snRNA in vertebrates: Evolutionary conservation of 5′ sequences implicated in splicing of pre-mRNAs containing a minor class of introns. *RNA 1*:644–656.

Waibel F, Filipowicz W. 1990. U6 snRNA genes of *Arabidopsis* are transcribed by RNA polymerase III but contain the same two upstream promoter elements as RNA polymerase II-transcribed U-snRNA genes. *Nucleic Acids Res 12*:341–348.

Wiebauer K, Herrero JJ, Filipowicz W. 1988. Nuclear pre-mRNA processing in plants: Distinct modes of 3′-splice-site selection in plants and animals. *Mol Cell Biol 8*:2042–2051.

Wolff T, Bindereif A. 1992. Reconstituted mammalian U4/U6 snRNP complements splicing: A mutational analysis. *EMBO J 11*:345–359.

Wu H-J, Gaubier-Comella P, Delseny M, Grellet F, Van Montagu M, Rouzé P. 1996. Non-canonical introns are at least 10^9 years old. *Nat Genet 14*:383–384.

Yean S-L, Lin R-J. 1991. U4 small nuclear RNA dissociates from a yeast spliceosome and does not participate in the subsequent splicing reaction. *Mol Cell Biol 11*:5571–5577.

Yu Y-T, Steitz JA. 1997. Site-specific crosslinking of mammalian U11 and U6atac to the 5′ splice site of an AT-AC intron. *Proc Natl Acad Sci USA 94*:6030–6035.

Yu Y-T, Tarn W-Y, Yario TA, Steitz JA. 1996. More Sm snRNAs from vertebrate cells. *Exp Cell Res 229*:276–281.